

AD \_\_\_\_\_

GRANT NO: DAMD17-94-J-4445

TITLE: Pleiotrophin as a Growth Factor and Therapeutic Target in Breast Cancer

PRINCIPAL INVESTIGATOR(S): Anton Wellstein, Ph.D., M.D.

CONTRACTING ORGANIZATION: Georgetown University  
Washington, DC 20057

REPORT DATE: October 1995

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

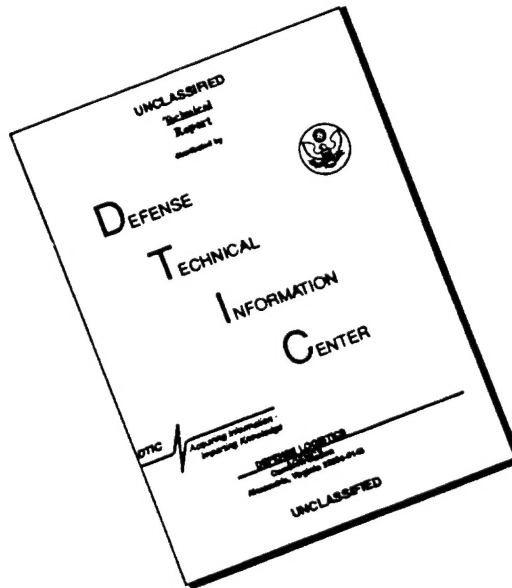
DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

19960621 116

NOT FOR PUBLICATION

# DISCLAIMER NOTICE



THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

# REPORT DOCUMENTATION PAGE

Form Approved

OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE October 1995		3. REPORT TYPE AND DATES COVERED Annual 15 Sep 94 - 14 Sep 95	
4. TITLE AND SUBTITLE Pleiotrophin as a Growth Factor and Therapeutic Target in Breast Cancer				5. FUNDING NUMBERS DAMD17-94-J-4445	
6. AUTHOR(S) Anton Wellstein, Ph.D. , M.D.					
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Georgetown University Washington, DC 20057				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES					
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited				12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)  We studied the effect of different hormones on pleiotrophin (PTN) expression in estrogen-independent, PTN-positive breast cancer cells. We found that retinoic acid upregulates and dexamethasone downregulates PTN mRNA. In addition we expressed the PTN cDNA in PTN-negative breast cancer cells and did not find a phenotypic change <i>in vitro</i> . Furthermore, we generated different single cysteine-targeting mutations in the PTN protein and found that the activity of the protein was not affected by a single change in a disulfide bridge. Finally, we assessed the effects of antisense oligonucleotides, antisense mRNA and of ribozymes on the phenotype of PTN-dependent model cell lines. We found that antisense oligonucleotides can inhibit PTN expression in PTN-positive cells and can slow the initial growth of a PTN-positive cell line in a xenograft model. However, the selectivity of the antisense oligonucleotides was only small (3-fold). Furthermore, we found that antisense transcripts can inhibit PTN expression and slow tumor growth by approximately 50% of a PTN-dependent model cell line in the athymic nude mouse model. Finally, we generated specific, PTN-targeted ribozymes that can reduce PTN mRNA in PTN-positive cells with high efficacy. The growth phenotype of a PTN-dependent model cell line was changed by the ribozyme targeting. In conclusion, we have generated a number of initial data and a series of molecular tools for our subsequent studies.					
14. SUBJECT TERMS growth factor, pleiotrophin, hormones, mutations, antisense, ribozymes breast cancer				15. NUMBER OF PAGES 18	
				16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited		

## GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to **stay within the lines** to meet **optical scanning requirements**.

### Block 1. Agency Use Only (Leave blank).

**Block 2. Report Date.** Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

**Block 3. Type of Report and Dates Covered.** State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

**Block 4. Title and Subtitle.** A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

**Block 5. Funding Numbers.** To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

C - Contract	PR - Project
G - Grant	TA - Task
PE - Program Element	WU - Work Unit Accession No.

**Block 6. Author(s).** Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

**Block 7. Performing Organization Name(s) and Address(es).** Self-explanatory.

**Block 8. Performing Organization Report Number.** Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

**Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es).** Self-explanatory.

**Block 10. Sponsoring/Monitoring Agency Report Number.** (If known)

**Block 11. Supplementary Notes.** Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

**Block 12a. Distribution/Availability Statement.** Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

**DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."

**DOE** - See authorities.

**NASA** - See Handbook NHB 2200.2.

**NTIS** - Leave blank.

### Block 12b. Distribution Code.

**DOD** - Leave blank.

**DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.

**NASA** - Leave blank.

**NTIS** - Leave blank.

**Block 13. Abstract.** Include a brief (*Maximum 200 words*) factual summary of the most significant information contained in the report.

**Block 14. Subject Terms.** Keywords or phrases identifying major subjects in the report.

**Block 15. Number of Pages.** Enter the total number of pages.

**Block 16. Price Code.** Enter appropriate price code (*NTIS only*).

**Blocks 17. - 19. Security Classifications.** Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

**Block 20. Limitation of Abstract.** This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the US Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

✓ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

W. H. L. C. 2-22-96  
PI - Signature Date

## TABLE OF CONTENTS

section	page
FRONT COVER	1
REPORT DOCUMENTATION PAGE	2
FOREWORD	3
TABLE OF CONTENTS	4
INTRODUCTION	5
BODY	6
CONCLUSIONS	17
REFERENCES	18

## INTRODUCTION

Under our proposal we study the role of the growth factor pleiotrophin (PTN) in breast cancer. We hypothesize that PTN is an essential, rate-limiting growth factor for PTN-positive breast cancers. Our experiments planned are designed to address this hypothesis.

We were the first laboratory to purify PTN from human cancer cells [1]. We were also the first laboratory to generate biologically active recombinant PTN [2] and to demonstrate its potential role as a tumor growth and angiogenesis factor [2]. Furthermore, we showed its distinct expression in human breast cancer samples [2] and published the genomic structure of the human PTN gene first [3].

We propose that the secreted polypeptide growth factor pleiotrophin (PTN) plays a major role in the growth and metastasis of breast cancer. This hypothesis is based on the high levels of PTN expression in 60% of tumor samples from breast cancer patients but not in normal tissues and on the biological effects of PTN in selected tumors models. In particular, the activity of PTN on endothelial cells indicates that PTN can serve as a tumor angiogenesis factor and its expression can thus enhance the ability of breast cancer to metastasize. In summary, our studies quoted above as well as the data published by others show:

- (1) PTN is a secreted growth factor expressed in a number of human breast cancer cell lines.
- (2) PTN stimulates endothelial cells and can act as a tumor angiogenesis factor (others: [4]).
- (3) PTN can support tumor growth of non-tumorigenic SW-13 cells (others: [5]).
- (4) PTN mRNA is upregulated by retinoic acid treatment of a PTN-positive breast cancer cell line.
- (5) PTN mRNA is found at high levels in 60% of samples from patients with breast cancer.
- (6) PTN is not expressed in normal breast epithelium ( 40% of the breast cancer samples were negative for PTN inspite of the fact that they obviously contain breast epithelial tissue).

### OVERVIEW OF THE GOALS:

In our studies, we elucidate the role of PTN and the hormonal regulation of its activity, with the ultimate goal to develop novel therapeutic strategies for breast cancer.

In particular, (1) we study to what extent hormonal regulation affects PTN expression as well as PTN-dependent tumor growth,

(2) investigate whether PTN expression can support hormone-independent growth of breast cancer and thus contribute to the resistance of tumors to treatment with anti-hormones,

(3) generate mutant PTN protein that can act as an inhibitor for wild-type PTN and

(4) target PTN mRNA with ribozyme, antisense constructs and oligonucleotides to repress production of PTN and inhibit breast cancer growth and metastasis.

### OVERVIEW OF METHODS:

For goals (1) and (2) steroid hormones and retinoids are used on breast cancer cells that have been transfected with a PTN expression vector. PTN-positive (goal 1) and PTN-negative (goal 2) cell lines are used.

For goal (3) mutation analysis and assays for activity of mutant PTN are used.

For goal (4) different constructs blocking PTN production are used.



## BODY

### Specific Aim (1):

**Function and regulation by hormones of pleiotrophin (PTN) in PTN-positive breast cancer cells.**

#### Background:

Hormones and growth factors define the capacity of human breast cancer to grow and metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [6]).

#### Work accomplished:

##### 1. Hormones and PTN in PTN-positive cells:

We tested MDA-MB 231 estrogen-independent human breast cancer cells that are positive for PTN for the regulation of PTN by hormones. **All-trans-retinoic acid** upregulated after 4 days the level of PTN mRNA in these cells by 10-fold. After 24 hours no upregulation was observed. We tested the growth response of PTN-positive MCF-7/ADR and T-47D/CO cells to all-trans retinoic acid and found that both cell lines are growth inhibited completely within 4 days of treatment and approximately 50% after 3 days. We do not yet have the data on the regulation of PTN in these cells at earlier time points. These experiments are ongoing.

The **glucocorticoid dexamethasone** was also used in MDA-MB 231 cells. The dose-response curve on PTN mRNA levels and on protein levels is shown in the figure on the next page and shows complete downregulation of the mRNA and protein by dexamethasone with a half-maximally effective concentration of 3 nM. Based on this, this downregulation appears to be receptor-mediated.

The treatment with dexamethasone was for five days and we cannot judge whether the downregulation is a direct effect on transcriptional or posttranscriptional regulation of PTN. We are currently running time-response curves with 0.1  $\mu$ M of dexamethasone.

Dexamethasone experiments are ongoing with the other PTN-positive human breast cancer cells mentioned above to assess whether the dexamethasone effect is a more general phenomenon or only apparent in the MDA-MB 231 cells.

**Sex steroids:** Experiments with the MCF-7/ADR and T-47D/Co cells are ongoing.

**Conclusion:** We judge that the downregulation of PTN by dexamethasone is a particularly exciting result since we now have two defined hormonal pathways that can regulate this gene product either up (retinoic acid) or down (dexamethasone) and interaction studies between the two hormones will be worthwhile in MDA-MB 231 cells.



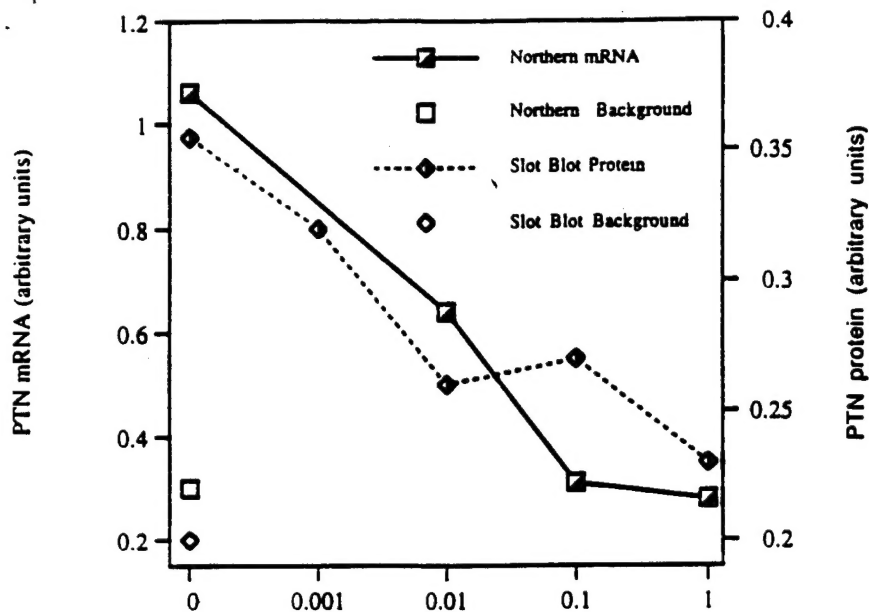


Figure 1

Dexamethasone (μM)

**Dose Response of Dexamethasone on PTN mRNA and Protein in MDA MB 231 Human Breast Cancer Cells in vitro.**

MDA MB 231 cells were treated with dexamethasone at the concentration indicated for five days. On day four, media was exchanged for fresh media containing dexamethasone. On day five cells and conditioned media were collected. PTN mRNA was detected by Northern analysis and PTN protein was analyzed by slot blot. The data from one representative experiment is shown along with the corresponding background on the Northern, as well as the slot blot. Values in arbitrary units of densitometry.

## 2. Transfection of PTN into PTN positive cells

We transfected MDA-MB 231 cells (PTN-positive and estrogen-independent) with an expression vector for PTN for future *in vivo* studies planned with retinoids and dexamethasone. To date only few drug-resistant clones grew up and initial test show that they do not express the transfected gene at high levels. We plan to repeat the transfection and selection with different transfection methods.

## 3. In vivo studies

No in vivo studies have been initiated yet.

### Problems & solutions:

We had problems obtaining sufficient numbers of clones from MDA-MB 231 cell lines after transfection with a drug resistance gene (G-418). We will repeat the transfections with different methods of gene transfer.

### Next steps:

The next steps include studying effects of retinoic acid and dexamethasone on the two other PTN-positive breast cancer cell lines, running time courses of the action as well as interaction studies of the hormones. Furthermore, the sex steroid studies will be brought to a conclusion.

**Specific Aim (2):**

**To study the effect of expression of PTN on the malignant phenotype of PTN-negative breast cancer cells.**

**Background:**

Hormones and growth factors define the capacity of human breast cancer to grow and ultimately to metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [6]).

**Work accomplished:**

**1. Transfection of PTN into PTN-negative breast cancer cells:**

We have transfected PTN-negative, estrogen-dependent MCF-7 wild-type and T-47D wild-type human breast cancer cells with an expression construct for PTN (see [2]) and have generated a series of different cell lines (mass-transfected and some clonal cell lines) expressing PTN. We have tested the cells *in vitro* for their proliferation and colony forming abilities as well as for expression of PTN mRNA and the secretion of protein.

A wide range of expression levels of PTN was achieved in different MCF-7-derived and T-47D-derived cell lines. No gross difference in the *in vitro* phenotype of the cells was observed. No significantly different proliferation on plastic surface or colony forming ability was found. Based on the current data we conclude that PTN is not utilized by T-47D or by MCF-7 cells as an autocrine growth factor. However, these *in vitro* experiments need to be expanded with hormone and anti-hormone addition to challenge the cells.

Currently, the further *in vitro* analysis of these cell lines is underway and we plan to initiate animal tumor growth studies after completion of the *in vitro* analysis.

We have not generated ZR-75 cell transfectants yet.

**2. Animal studies:**

We have not initiated animal studies since we have not completed the *in vitro* analysis.

**Next steps:**

We plan to complete the *in vitro* analysis of the growth characteristics and hormone effects on transfected cells.

**Specific Aim 3:**  
**The function of the different domains of the PTN protein**

**Background:**

The secreted PTN protein contains two distinct cysteine-rich domains (on two separate exons) that contains three and two disulfide bridges respectively. Disulfide bridge formation is required for biological activity of the protein. We hypothesize that defined mutations will generate a protein that can still bind to the receptor but will fail to activate the receptor and can thus serve as an antagonist.

**Work accomplished:**

We have generated point mutant PTNs that have the N-terminal or the C-terminal cysteine changed to a serine and thus disrupted disulfide bridge formation. We tested the effects of the mutant proteins in transfection assays using expression vectors for the mutant and for wild-type PTN in PTN-responsive SW-13 cells as indicator cells of activity.

To our astonishment we found that the N- or C-terminal cysteine mutations affect the activity in transient transfection assays only very little. Furthermore, the amount of protein secreted is not decreased suggesting that the mutant proteins are as stable as the wild-type protein. We conclude from this finding that the N-terminal and the C-terminal disulfide bridge are not essential for stability and activity of the protein when only one of them is destroyed. We decided to go ahead with double mutants that will mutate both disulfide bridges at the same time. Characterization of these constructs is ongoing.

**Problems and solutions**

We produced recombinant wild-type and mutant protein from supernatants of COS-7 cells for further analysis. However, we found that the wild-type protein shows very little if any activity most likely due to improper processing in the cells and we reverted to the production of wild-type and mutant proteins in SW-13 cells in which we can test the activity at the same time.

**Next steps**

We will test the stability and activity of the double-cysteine mutant protein in transient transfection assays and plan to delete and replace the cysteine-rich domains in the next round of vector generation.

**Specific Aim 4:**

**To inhibit production of PTN by antisense (AS) and ribozyme (RZ) constructs and to develop antisense oligonucleotides as novel drugs for breast cancer therapy.**

**Background**

We planned to use three independent approaches to target PTN mRNA and thus reduce the amount of PTN produced by PTN-positive breast cancer cells:

1. antisense oligonucleotides
2. antisense constructs
3. ribozyme constructs

**Work accomplished**

**1a. antisense oligonucleotides *in vitro***

We selected phosphorothioate-antisense oligonucleotides that are targeted to the PTN open reading frame. We used SW-13/PTN-transfected cells as our model [2] to assess the efficacy and specificity of the approach. The figure below shows the dose-response of solvent (control), scrambled and antisense oligonucleotides in these cells. As a read-out we used their colony formation in soft agar since we had established that earlier as a specific effect of PTN transfection into the cells.

As the data in the figure on the next page show, an approximately 3-fold selectivity between toxic effects by the scrambled oligonucleotide and specific effects of the antisense oligonucleotide can be achieved. This was confirmed by PTN protein assay of the cell supernatants (not shown). This was somewhat disappointing since we had hoped for higher selectivities of the compounds.

**1b. treatment with antisense oligonucleotides *in vitro* and *in vivo* measurement of the effect on tumor growth**

Although the selectivity of the antisense oligonucleotides was not very encouraging, we decided to run a pilot study to assess whether we could prove an effect of the treatment *in vitro* on tumor formation *in vivo*. We found that a single pretreatment of SW-13/PTN cells with the antisense oligonucleotides *in vitro* and subsequent wash-out of the oligonucleotides kept the PTN protein down for approximately one week. After that the cells would have eliminated the oligonucleotides and resume production of the PTN protein. We hypothesized that a pretreatment of cells with antisense oligonucleotides *in vitro* and subsequent inoculation of tumor cells into animals would thus generate a delay of tumor growth for approximately one week, if PTN was a rate-limiting factor for tumor growth. If PTN was not rate-limiting no effect would be seen. Also, if the tumors grew very slowly, the effect would be difficult to prove due to the scatter observed during initial tumor growth of xenografted cells. With this in mind, we opted to run a pilot study with a tumor cell line that expressed high levels of PTN and forms detectable tumors in athymic nude mice within a few days after inoculation. The data from this experiment are shown in the figure two pages.

We interpret the data to mean that the anti-sense oligonucleotide pretreatment works and that PTN is rate-limiting for tumor growth of these cells in animals. We are considering a similar experiment with PTN-positive breast cancer cells. One way to circumvent the slow onset of growth into tumors of these cells, we plan to use a high initial tumor cell load for inoculation.

# Colony formation in soft agar of transfectant SW-13/PTN cells

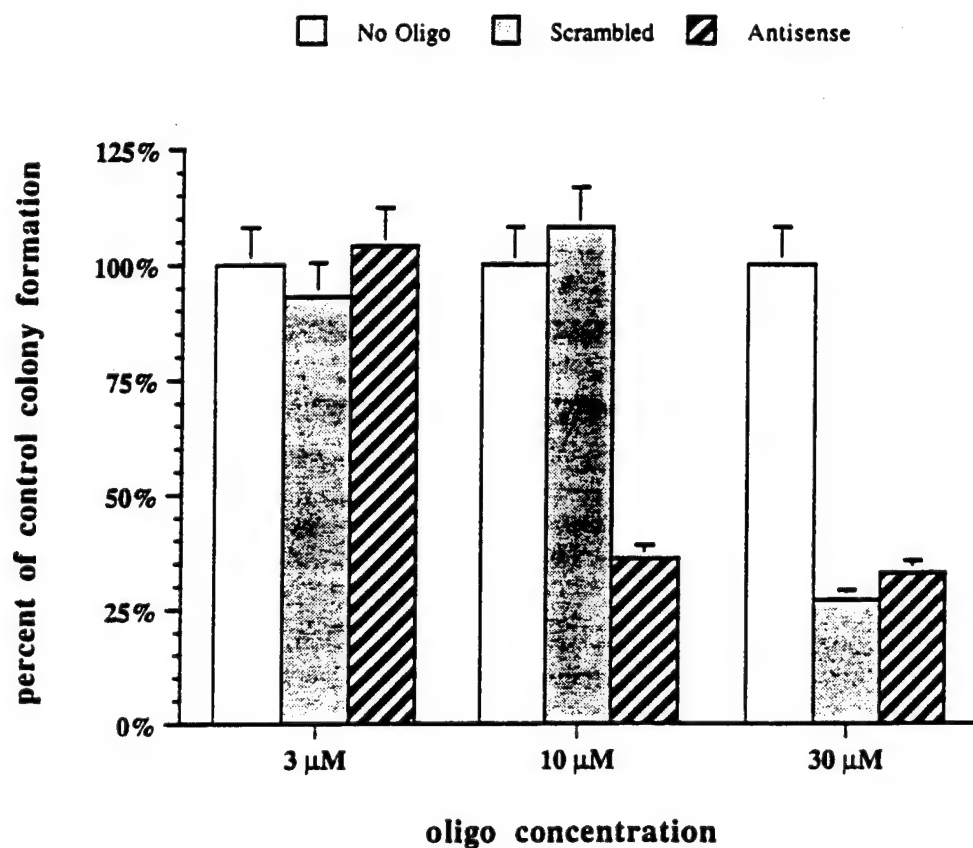


Figure 2

Effect of oligonucleotides on PTN-transfected SW-13 cells: Colony formation in soft agar of antisense-treated transfectant SW-13/PTN cells.

### Tumor Formation in Athymic Nude Mice

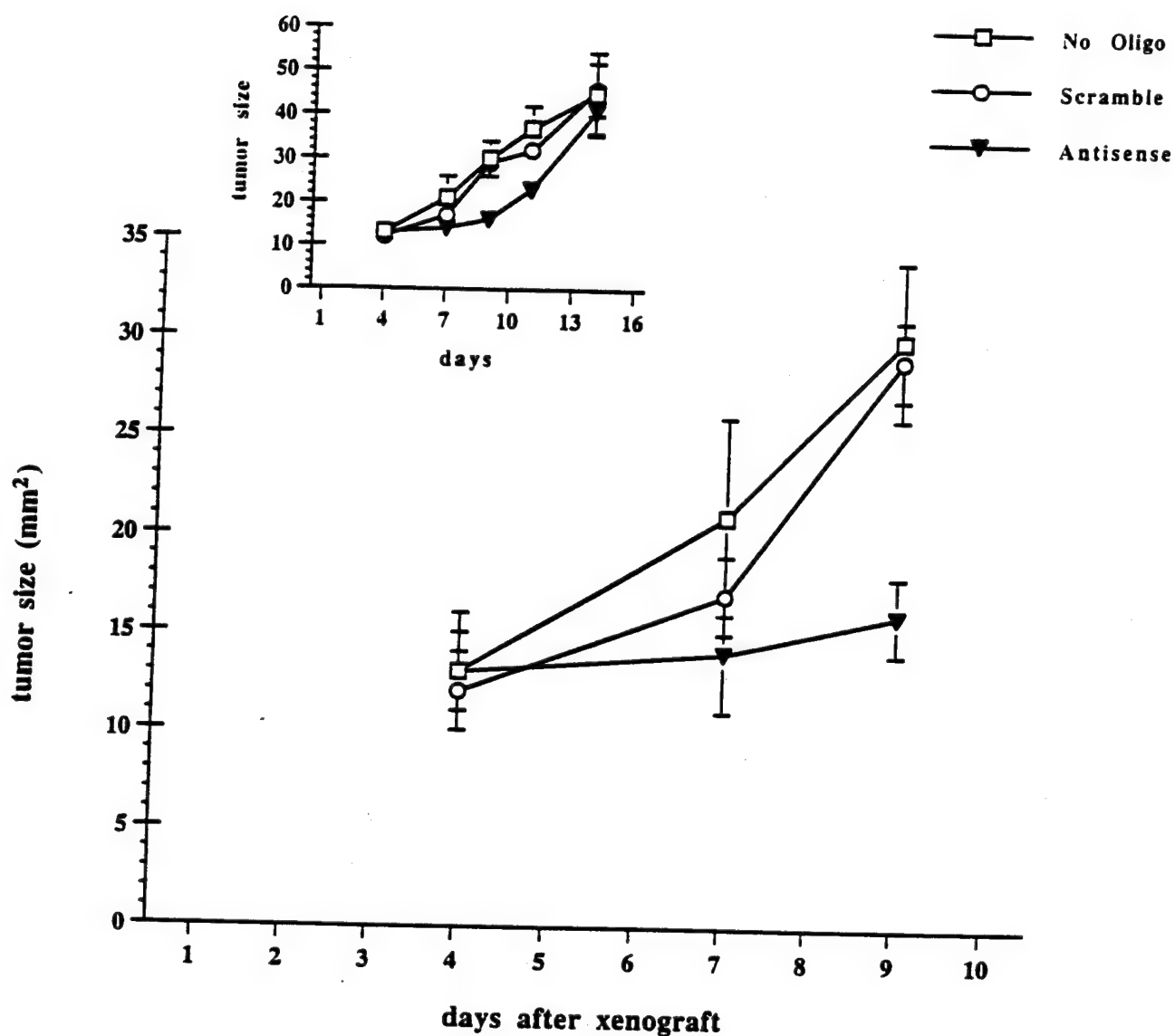


Figure 3

Effect of oligonucleotides on 1205-LU melanoma cell tumor growth in athymic nude mice: 1205-LU melanoma cells were injected subcutaneously into nude mice after pretreatment with oligonucleotides for 72 hours.

## 2. antisense constructs; measurement of the effect on tumor growth

In addition to antisense oligonucleotides, we proposed to generate different antisense constructs to target PTN. Five sequences ranging in length from 103 to 509 nucleotides, chosen to overlap with the PTN regions previously targeted by the antisense oligonucleotides, were amplified by PCR from the pleiotrophin open reading frame using the pRc-CMV/PTN plasmid [2] as a template. The amplified fragments, as described in Table 1 below were denoted ptnAS1 (108 nt), ptnAS2 (252 nt), ptnAS3 (103 nt), ptnAS4 (277 nt) and ptnAS5 (509 nt). They were verified for size by agarose gel electrophoresis. A sufficient yield of all five fragments was obtained after PCR.

**Table 1:**

DNA fragments amplified from the PTN open reading frame used to construct antisense vectors:

<u>Fragment</u>	<u>Abbreviation</u>	<u>Region</u>	<u>Length (nt's)</u>
5' short	AS1	-12 to +96	108
5' long	AS2	-12 to +240	252
3' short	AS3	+395 to +497	103
3' long	AS4	+221 to +497	277
Largest	AS5	-12 to +497	509

The fragments were then each ligated into a cloning vector, amplified in *E. Coli*, selected for transformed clones and further selected for clones which contained antisense orientation DNA fragments. This multi-step process yielded four of the five targeted antisense vector clones, but did not yield ptnAS4. This was because during this procedure the yields of ptnAS4 (3' long fragment) were diminished enough at certain steps such that further generation of any ptnAS4 plasmids was undermined. The four remaining antisense fragments were subsequently excised from the cloning vector using the restriction enzymes HindIII and XbaI, low-melting agarose gel-purified, subcloned into the pRc/CMV vector, and sequenced. As verified by sequencing on 6% PAGE, four constructs (ptnAS1, ptnAS2, ptnAS3 and ptnAS5) contained intact and proper antisense regions at the cloning site.

After eventual propagation in bacteria and preparation of plasmids, antisense vectors pRc-CMV/AS1 (containing ptnAS1) and pRc-CMV/AS3 (containing ptnAS3) were obtained in sufficient quantities for transfection into the model cell line used in the *in vitro* / *in vivo* experiment above. Antisense vectors pRc-CMV/AS2 (containing ptnAS2) and pRc-CMV/AS5 (containing ptnAS5) were not obtained in as high yields as the aforementioned two and were frozen for potential future studies. The two antisense vectors (ptnAS1 and ptnAS3) cover the translation initiation site (AUG) and a putative loop-forming region in the 3' open-reading frame of PTN. Additionally, each of these vectors respectively coincides with the PTN regions targeted by antisense oligonucleotides and ribozymes which proved to be effective in independent studies presented above and discussed further below.

We conclude from the data shown below, that antisense transfection is effective in this model of tumor growth. However, the effect on tumor growth is only partial. This could mean a lack of efficacy of the antisense transfection or that PTN is not the rate-limiting growth factor *in vivo* for this tumor cell line. Other independent studies with ribozymes in this cell line (unpublished data) suggest that PTN is the rate-limiting factor for this tumor cell line and we conclude from that on the lack of full efficacy of the antisense transfection studies.



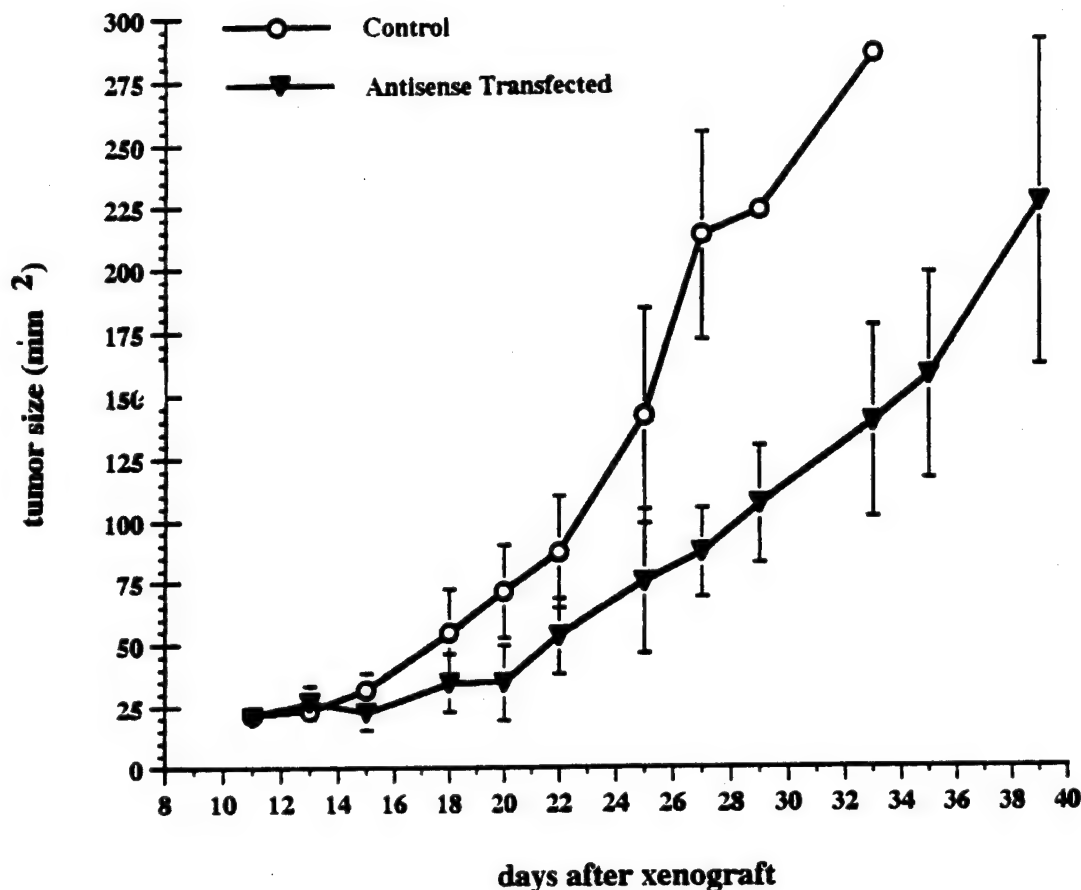


Figure 4

Effect of stable PTN antisense transfection on 1205-LU cell tumor growth in athymic nude mice: 1205-LU control, 1205-LU/AS1 and 1205-LU/AS3 melanoma cells were injected subcutaneously into athymic nude mice. Tumor size data from the antisense-transfected tumors were pooled, since they did not show any difference in growth rates.

### 3. ribozyme targeting of PTN

We have completed the generation and testing of different ribozyme constructs that can inhibit PTN production in a model melanoma cell line. This work was completed early in 1994 and published in Aug. 94 before the initiation of this award in J. Biol Chem. [7]. The data relevant for our experiments in breast cancer under this award are discussed and shown below.

The principle of ribozyme-targeting is that an RNA-derived RNase (the ribozyme) is targeted to a specific region in the gene transcript of interest. This is achieved by generating a catalytic core structure of the ribozyme and by targeting it to a defined substrate by virtue of anti-sense flanking sequences on either side of the catalytic core. These ribozymes contain the same hammerhead structure that is derived from the original Haseloff-Gerlach ribozyme [8] and was modified and minimized to a 22 nucleotide catalytic center based on the mutational analysis that had shown the essential elements for a catalytically active RNA [9]. As illustrated for our Rz261 (see Fig. 1) the molecule should form three double helices ("hammerhead") which meet at the targeted cleavage site. In addition, two stretches of nucleotides with the sequences 5'-CUGAXGA and 5'-GAAA

(X is either A, U or C) are required for the catalytic activity. The cleavage is directed to a specific site in the targeted RNA by two stretches of 8 to 12 nucleotides each that flank the catalytic center on its 3'- and 5'-side. These flanking sequences hybridize with the targeted RNA by Watson-Crick base pairing (reviewed in ref.[10]). The catalytic activity of an active ribozyme and of a point-mutant ribozyme is shown in Fig. 2.

We inactivated the PTN gene by transfections with different hammerhead ribozyme constructs as described above (see also Refs. [8,10,11]) and studied the resulting phenotype of cells. In a PTN-responsive cell line (SW-13 [1,2]), co-transfection of PTN and of ribozymes specifically prevented the PTN-induced growth of the cells (published in [7]). A catalytically inactive, point-mutant ribozyme [9,12] was inactive in this assay. In human melanoma cells that constitutively express high levels of PTN mRNA, stable transfection with PTN-targeted ribozymes quenched production of PTN, inhibited colony formation of the melanoma cells in soft agar and prevent their growth into tumors in athymic nude mice [7]).

These data indicate that ribozymes are excellent tools to pinpoint the role of PTN. They will be extremely useful in our studies on PTN expressed in breast cancer cells.

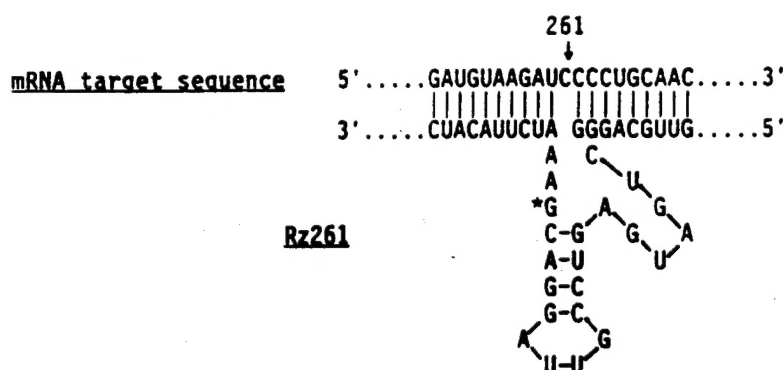


Figure 5

Predicted structure of hammerhead ribozymes used in our studies and the predicted hybridization with the targeted sequence in PTN flanking nucleotide # 261 downstream of translation initiation of PTN. Details published in Ref. [7].

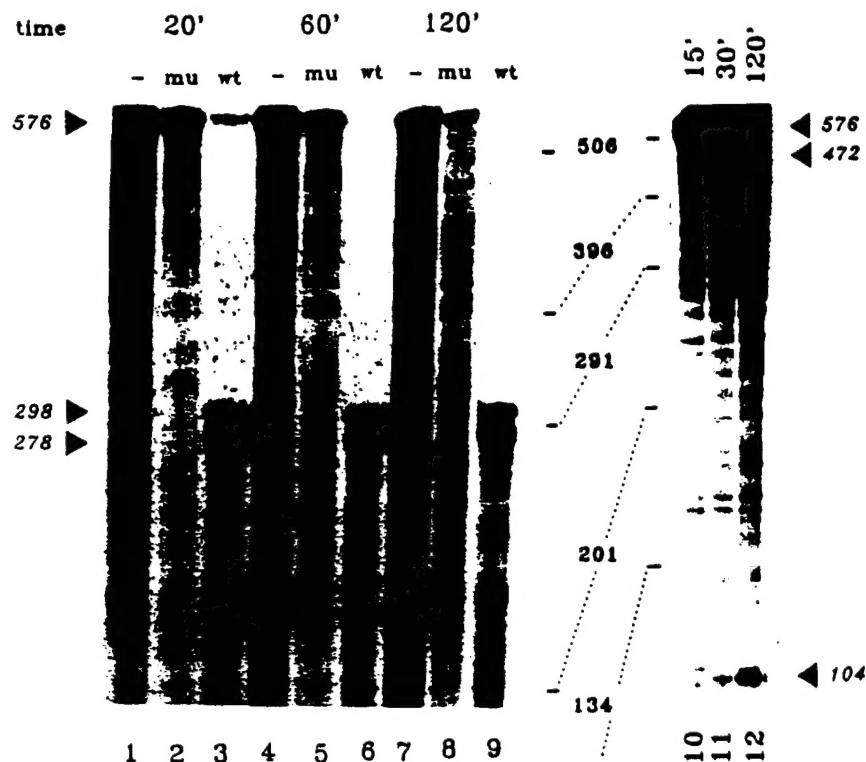


Figure 6

*In vitro* cleavage of a labelled PTN molecule by different ribozymes (Rz). Left panel: PTN was incubated for the indicated times alone (lanes 1,4,7) or together with mutant Rz261 that is inactive (lanes 2,5,8) or wild-type Rz261 that is active (lanes 3,6,9) and the products separated on a sequencing gel and autoradiographed. Right panel: PTN was incubated with the active Rz 66 for the indicated times. Details in Ref. [7].

#### Problems and solutions

The initial studies with antisense oligonucleotides were disappointing with respect to the low selectivity of the compounds. The antisense transfections were not fully effective in a model that showed full effect of ribozyme-targeting. On the other hand the experience with the ribozyme targeting is very encouraging for the studies in breast cancer. We have initiated transfections of MDA-MB 231 cells (PTN-positive breast cancer cells). In a first series of experiments the expression levels for the ribozymes seemed to have been too low. We will repeat this part of the experiments and perhaps pick clonal cell lines.

#### Next steps

Transfections of PTN-positive human breast cancer cells with PTN-targeted ribozymes are planned as a next round of experiments.

## CONCLUSIONS

We studied the effect of different hormones on pleiotrophin (PTN) expression in estrogen-independent, PTN-positive breast cancer cells. We found that retinoic acid upregulates and dexamethasone downregulates PTN mRNA. In addition we expressed the PTN cDNA in PTN-negative breast cancer cells and did not find a phenotypic change *in vitro*. Furthermore, we generated different single cysteine-targeting mutations in the PTN protein and found that the activity of the protein was not affected by a single change in a disulfide bridge. Finally, we assessed the effects of antisense oligonucleotides, antisense mRNA and of ribozymes on the phenotype of PTN-dependent model cell lines. We found that antisense oligonucleotides can inhibit PTN expression in PTN-positive cells and can slow the initial growth of a PTN-positive cell line in a xenograft model. However, the selectivity of the antisense oligonucleotides was only small (3-fold). Furthermore, we found that antisense transcripts can inhibit PTN expression and slow tumor growth by approximately 50% of a PTN-dependent model cell line in the athymic nude mouse model. Finally, we generated specific, PTN-targeted ribozymes that can reduce PTN mRNA in PTN-positive cells with high efficacy. The growth phenotype of a PTN-dependent model cell line was changed by the ribozyme targeting.

In conclusion, we have generated a number of initial data and a series of molecular tools for our subsequent studies. We believe that the research has gone at a good pace and that some of the data will be submitted for publication in the second year of the award.

## REFERENCES

1. Wellstein A, Fang WJ, Khatri A, Lu Y, Swain SS, Dickson RB, Sasse J, Riegel AT, Lippman ME: A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. *J Biol Chem* 267: 2582-2587, 1992
2. Fang WJ, Hartmann N, Chow D, Riegel AT, Wellstein A: Pleiotrophin stimulates fibroblasts, endothelial and epithelial cells, and is expressed in human cancer. *J Biol Chem* 267: 25889-25897, 1992
3. Lai SP, Czubayko F, Riegel AT, Wellstein A: Structure of the human heparin-binding growth factor gene pleiotrophin. *Biochem Biophys Res Commun* 187: 1113-1122, 1992
4. Courty J, Dauchel MC, Caruelle D, Nguyen TT, Barritault D: Purification and characterization of a new endothelial cell growth factor named HARP (Heparin Affin Regulatory Peptide). *J Cell Biochem* 15F: Abstr. 221, 1991(Abstract)
5. Chauhan AK, Li YS, Deuel TF: Pleiotrophin transforms NIH 3T3 cells and induces tumors in nude mice. *Proc Natl Acad Sci U S A* 90: 679-682, 1993
6. Dickson R, Lippman ME: Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. *Endocr Rev* 8: 29-43, 1987
7. Czubayko F, Riegel AT, Wellstein A: Ribozyme-targeting elucidates a direct role of pleiotrophin in tumor growth. *J Biol Chem* 269: 21358-21363, 1994
8. Haseloff J, Gerlach WL: Simple RNA enzymes with new and highly specific endoribonuclease activities. *Nature* 334: 585-591, 1988
9. McCall MJ, Hendry P, Jennings PA: Minimal sequence requirements for ribozyme activity. *Proc Natl Acad Sci U S A* 89: 5710-5714, 1992
10. Symons RH: Small catalytic RNAs. *Annu Rev Biochem* 61: 641-671, 1992
11. Uhlenbeck OC: A small catalytic oligoribonucleotide. *Nature* 328: 596-600, 1987
12. Sheldon CC, Symons RH: Mutagenesis analysis of a self-cleaving RNA. *Nucleic Acids Res* 17: 5679-5685, 1989